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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) **International Patent Classification** 6 : **A61K 31/28, 9/127**

A1

(11) **International Publication Number:** **WO 98/24425**

(43) **International Publication Date:** 11 June 1998 (11.06.98)

(21) **International Application Number:** PCT/NL97/00661

(22) **International Filing Date:** 3 December 1997 (03.12.97)

(30) **Priority Data:**
PCT/NL96/00474 3 December 1996 (03.12.96) WO
(34) Countries for which the regional or international application was filed: NL et al.

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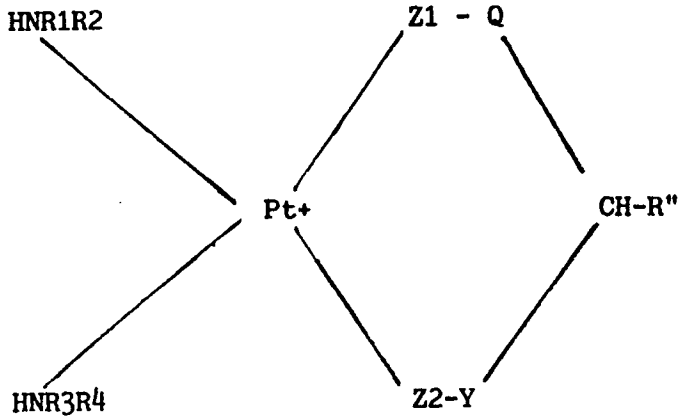
(81) **Designated States:** AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) **Title:** CISPLATINUM COMPRISING PHARMACEUTICAL

(57) **Abstract**

A pharmaceutical composition for use in treatment of a subject having neoplasms with a view to reducing size and/or number of neoplasms, said pharmaceutical composition comprising a complex of a cytostatic compound of the cis platinum family and an anionic organic compound as a complexed form of the active pharmaceutical component, said complex having general formula (1) wherein Q = –CO– or –PO(O⁻)–; Y = –CHR'–, –CO–, –PO(O⁻)– or a direct bond; Z1, Z2 = independently O or NR', R1, R2=H or a hydrocarbon group optionally substituted, optionally linked to each other or to R3 and/or R4 wherein R3, R4=H or hydrocarbon group optionally substituted, optionally linked to each other or to R1 and/or R2, wherein R'=H, or a hydrocarbon group optionally substituted and wherein R''=hydrocarbon group substituted by a group capable of liposome formation.



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CISPLATINUM COMPRISING PHARMACEUTICAL.

Summary of the invention

The subject invention lies in the field of pharmaceuticals. In particular the invention is concerned with a novel pharmaceutical composition for treatment of neoplasms. The novel pharmaceutical is a complexed form of a component belonging to the family of cisplatinum compounds. Particularly suitable embodiments comprise a complex of 1,2-diacyl-sn-glycero-3-phosphatidylserine with cisdiammineplatinum(II). The novel form should ensure reduced toxicity. In a preferred embodiment the pharmaceutical composition has a slow release form such as a micelle or liposome structure. The pharmaceutical composition according to the invention ensures a reduction of the severe side effects associated with cisplatinum treatment.

Background of the invention

Since the discovery of it's cytostatic activity in 1964 and 1969 the compound cis-$[PtCl_2(NH_3)_2]$ which is clinically known as cisplatin has become a very succesful antitumour drug[I.1]. Cisplatin is regularly used for treatment of testicular and ovarian cancer. The survival rate for testicular cancer is over 95% thanks to this drug. It also finds application in treatment of other cancer forms like head and neck tumours, cervical cancer and bladder tumours [I.2]. Recently lung tumors have also been treated. Cisplatin is one of the top three selling antitumor agents world wide.

It is also known that cisplatin forms adducts with many biological molecules but strong evidence suggests its principal target is DNA [II.2]. It was determined in early mechanistic studies reviewed in [I.2 and I.3] that cisplatin specifically binds to guanine N^7 atoms of DNA. There is good evidence that after administration either by injection/infusion or orally that the drug circulates in the blood. Primarily this will occur as the chloride (for cisplatin) due to the rather high concentration of chloride ions in blood. Relatively slow hydrolysis occurs(t1/2 of a few hours at 37°C) upon passing through cell membranes of both normal and tumor cells either inside the cell or during passsage into the cell [III.11] followed by binding to nucleic acids and probably many other targets. It has been found with in vitro competition tests between DNA and proteins that the degree of binding to DNA (guanine

N7) and S donor atoms is determined by the degree of hydrolysis of cis-Pt. Although the major target seems to be nucleic acid many low molecular weight competitors for cisplatin-DNA reactions in the cell are known and intracellular reactions with peptides and proteins may take place. Examples are Cl-, $(HPO_4)^{2-}$, OH-, H_2O, His, Met, Cys, gluthathione, metallothionein, ATP, amino acids and polyphosphates. The sites at proteins are believed to be the most likely origin of the several toxic side effects of cis-Pt and its several derivatives [III.5,10 and 14]. Significant amounts of administered cis-Pt are lost due to binding to proteins.

The disadvantage of antitumor agents that target nucleic acid is that not only tumor cells but also other healthy cells that also replicate at a high rate are automatically targeted. Examples are the inner lining of the stomache thus causing nausea, the white and red blood cells thus causing anaemia and fatigue, liver and kidney cells thus causing liver damage and nephrotoxicity. It is often these side effects that restrict the physician with regard to administration of more of the often extremely reactive cytostatic compound. Toxic side effects of cisplatin are severe and have stimulated a lot of research into derivatives of cisplatin.

The toxic side effects have also resulted in the development of special protocols regarding drug dosage [I]. By slow administration of the drug e.g. via infusion lasting several hours rather than administration of a single bolus a high toxic peak concentration in blood can be avoided. In addition the use of rescue agents like sodium dithiocarbamate has been developed in order to counter toxicity by rescuing the cis-Pt bonds to proteins as described above. An example is thiourea [III.15].

Derivatives belonging to the cisplatinum family of compounds that are capable of similar or improved biological activity are known. Up to now only 10-15 have reached clinical trials. The cis platinum family of compounds comprises compounds of the structural class cis-$[PtX_2(amine)_2]$, wherein X is the leaving anionic group and amine is any primary or secondary amine. It has been possible to formulate structure-activity relationships for platinum compounds. The cis-geometry of two amines and the presence of at least one N-H group on the amine as well as the leaving groups with a weaker trans effect than the amine have been found to be necessary. The amines can be symmetric, asymmetric, chelating or nonchelating. Water solubility should be good and toxic side effects

should be minimised. Possible reactions in the blood with ligands containing S donor atoms should be suppressed [I].

New platinum complexes containing tissue specific carrier molecules as ligands for achieving higher concentrations have been described(I.7]. New platinum complexes attached to other chemotherapeutic agents like intercalators [I.8] or phosphonocarboxylates[I.9] as coligands to obtain some sort of synergistic effect have been disclosed. Alternative compounds have more than one platinum atom connected by a bridge [I.10], contain radiosensitizers as ligands [I.11] and have protecting groups released by antibody linked enzymes only at the surface of the specific tumour cells [1.12].

The second generation platinum drug carboplatin, $[Pt(C_6H_6O_4)(NH_3)_2]$ has been developed and is in routine use. This compound has less toxic side effects than cisplatin. It has a lower reactivity which allows a higher dose to be delivered. The dosage of carboplatin can be up to 2000mg/dose[I.2]. The cisplatin dosage is usually around 100 mg/day for up to five consecutive days. The second generation drugs are generally referred to as CBDCA derivatives after the parent compound.

Beside the classical platinum compounds completely different structural classes of platinum compounds have also been developed. These include Pt(IV) and dinuclear compounds. In short a great amount of research in lots of different directions has been carried out around cisplatinum derivatives in order to improve the pharmaceutical. The references [III. 10,11,12 and 13] provide examples of the cisplatinum compounds known to date and are incorporated herein by reference.

In order to reduce development of resistance to the cytostatic further derivatives, the socalled third generation derivatives have evolved. These include platinum(IV) derivatives that can be administered orally. An example of a third generation drug [I.5] is $[Pt(Cl_2(RCO_2)_2(amine)_2]$, wherein R=alkyl. Treatment to try and prevent resistance occurring comprises administering a number of chemotherapeutic agents rather than one and applying a varied scheme of administration. In particular upon treatment of ovarian cancer a major limitation of cisplatin is acquired resistance. The dose escalation required to overcome even a small increase in cellular resistance can cause severe toxicity.

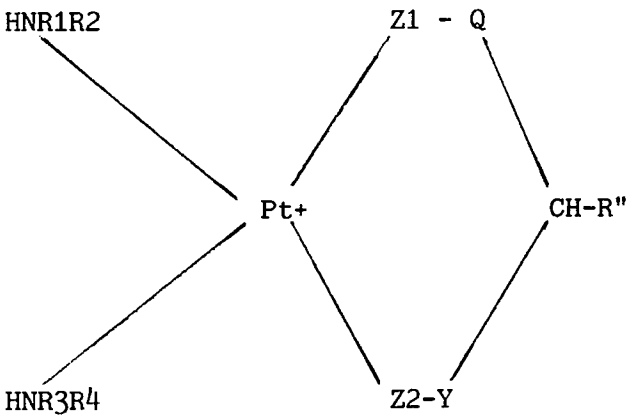
In summary as regrettably for the past 25 years with perhaps the exception of taxol no new strongly improved antitumor agents have been developed a lot of time and effort has gone into improving existing

therapies and administration methods. A lot of research into derivatives of cisplatin has been aimed at either reducing toxicity and/or circumventing resistance.

The subject invention is directed at formulating novel and improved cisplatinum derivatives. The improved pharmaceutical composition comprises a stable cis-platinum derivative in an inert form in the bloodstream. The novel composition thus exhibits reduced toxicity in the bloodstream. Additionally, loss of active compound due to binding of phosphatidylserine in vivo which can occur with the prior art compounds can now also be prevented. Furthermore upon entry into the cell the complexed form can be released thereby providing a cisplatinum derivative capable of becoming the most active form of the cytostatic. The Cl- concentration and pH and temperature in the bloodstream provide an environment ensuring stable binding of the cis-platinum compound such that it does not occur in the hydrolysed active form in the bloodstream to the degree the corresponding non complexed compound does. The Cl- concentration and/or presence of other components in the cell known to compete for cisplatinum binding provide the circumstances required for the cis-platinum derivative to be released in it's active form. The complex preferably releases cis-platinol which is considered to be the pharmacologically most active form for treating neoplasms directly in the cell. This form binds to nucleic acid and exerts it's activity. The pharmaceutical composition thus is present in an inactive form in the bloodstream thereby avoiding the toxicity effects due to the binding to phosphatidylserine. In addition the toxicity effects due to the presence of cisplatinol in the bloodstream common to the prior art compounds can also be avoided. In particular the complexed form can slowly release the active form in the bloodstream thereby avoiding plasma peaks of the extremely toxic cisplatinum compound in a form other than the complexed form according to the invention. Further advantage is provided by quick release of the pharmaceutically active cisplatinol becoming possible in a cell which is ultimately the target of the cytostatic compound. An essential aspect of the invention is the reversibility of the complex formation. The cisplatinum derivative must be releasable from the anionic organic binding partner.

The pharmaceutical composition according to the invention is a pharmaceutical composition for use in treatment of a subject having neoplasms with a view to reducing size and/or number of neoplasms, said pharmaceutical composition comprising a complex of a cytostatic compound

of the cis platinum family and an anionic organic compound as complexed form of the active pharmaceutical component, said complex having the following general formula 1



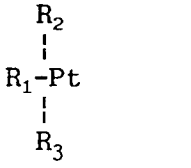
wherein

Q = -CO- or -PO(O^-)-;
Y = -CHR'-, -CO-, -PO(O^-)- or
a direct bond;
Z1, Z2 = independently O or NR';
R1,R2=H or a hydrocarbon group optionally substituted, optionally linked to each other or to R3 and/or R4
wherein R3,R4=H or hydrocarbon group optionally substituted, optionally linked to each other or to R1 and/or R2,
wherein any of the nitrogen atoms can be protonated or quaternised and wherein
R'=H, or a hydrocarbon group optionally substituted, and wherein
R"= a hydrocarbon group substituted with a group capable of liposome formation.

Liposome technology has been available for a number of years and has been the subject of enormous amounts of research. The cosmetic industry and also the pharmaceutical world have produced a number of useful products with this technology. Vaccines and other medicaments have been introduced into liposomes. Upon formation of a liposome with a correct composition the liposome is degraded slowly and the medicament is released after a delay. To date the only antitumor agent commercially available in a liposome is doxorubicin. The dosage can be ten times higher than without the liposome. Experiments are ongoing with

daunorubicin a related compound of doxorubicin. A vinca alkaloid has also been described as being present in a liposome. Generally speaking there are two options when forming liposomes capable of carrying medicaments. First the construction of a multilamellar bilayer vesicle with water comprised between the layers. The medicament is comprised within this water fraction. It is not linked to the membrane structure of the liposome but is captured in the water layer. Alternatively it has been possible to incorporate compounds with hydrophobic groups within the membrane structure of the liposome or attached to the membrane layer. European patent application EP-A-0.664.116 provides a method for producing liposomes wherein lecithin is used as a cell component. Lecithin consists of phosphatidylcholine and does not comprise phosphatidylserine. This publication suggests using such liposomes for delivering a large number of compounds. One of the compounds mentioned is cisplatin. However no illustration of this is provided. In fact such an embodiment as suggested would not be feasible. The cisplatin is a small hydrophilic molecule which is not expected to remain trapped in such a liposome structure as taught in the document for a sufficient length of time to provide a suitable slow release medicament. In WO96/15774 a process and apparatus for making liposomes containing hydrophobic drugs is disclosed using critical, supercritical or near critical fluids. Such structures comprise the drug enclosed within the liposome structure not as a structural component of the liposome itself. In addition the description mentions a large number of potentially useful hydrophobic drugs and includes in this list the compound B cisplatin. However, as already mentioned for EP-A-0.664.116, cisplatin is hydrophilic and will not complex to a component of the liposome under the conditions described, thus it will not form a compound according to the invention with slow release capability.

In WO90/02131 a platinum (II) four coordinant complex



(II) is disclosed wherein

R_1 is an alkyl diamine or cycloalkyl diamine and R_2 and R_3 are an alkylcarboxylato containing from five to ten carbon atoms. The document also suggests incorporation of such complex in a liposome. The compounds suggested in said document do not fulfil the above requirements. The dissociation behaviour of the ligands for the compounds suggested in

WO90/02131 will not be such to ensure a slow release of the active platinol in vivo of the order attained by the compounds according to the invention. The cited document is directed at compounds to be included in a liposome but not to be complexed to other components forming the liposomal structure. The compounds according to the invention will due to the presence of 2 ligands on one molecule exhibit far better dissociation characteristics for slow release. The fact that the complex forms a structural component of the liposomal structure as opposed to be merely being present within the liposomal structure by encapsulation also adds to the improved properties. In the subject compounds the groups corresponding to R_2 and R_3 of the cited document, i.e. Z_1-Q and Z_2-Y form a chelate, thereby ensuring higher stability and improved suitability for use as a prodrug. The chelated drug cbcda e.g. exhibits 100 x 150 times slower release than cisplatin as has been disclosed in an article by one of the inventors [Document I] and by Frey et al. [Document IV].

It is possible in alternative embodiments of the invention to provide unilamellar vesicles or multilamellar vesicles. Both embodiments are useful versions of a pharmaceutical composition according to the invention. It is also possible to produce micelles comprising the complex according to the invention thus providing a simple form of cytostatic exhibiting reduced toxicity. The substituent R" thus may have many embodiments which are obvious to a person skilled in the art of liposome formation or micelle formation. The requirements for the membrane components to produce a liposome suitable for application to a mammal are well known to a person skilled in the art as they are also for micelle formation. Such general information is readily available in general handbooks and text books on liposomes.

The pharmaceutical composition according to the invention suitably comprises as substituents R1,R2,R3 and R4 substituents common to what are generally known in the art as belonging to the cisplatinum family of compounds. These have been described in the introductory part of the description and are all incorporated herein by reference. In particular the first and second generation derivatives are suitable. Preferable compounds comprise cisplatinum substituents in combinations already in use clinically. The most preferred embodiment comprises the compound most widely used as cytostatic i.e. wherein the cytostatic compound is a cis diammineplatinum(II) derivative i.e. NR1R2=NR3R4=NH3.

A suitable embodiment of a pharmaceutical composition according to invention comprises as anionic organic compound the structure of

formula 1, wherein R" = -A-O-SO_2-O-R"' or preferably R" = -A-O-PO_2^--O-R"' wherein A=CnH2n, n=1-4, optionally substituted with hydroxy or phenyl; R"'=H, or a hydrocarbon group such as alkyl, alkenyl, optionally substituted by amino, hydroxy, acylamino and/or acyloxy. The R"' can comprise one or more hydrocarbon group chains. The hydrocarbon group chains can be substituted or non substituted. It can comprise one or more alkyl, alkenyl, amino, hydroxy, acylamino and/or acyloxy chains or a combination thereof. It is e.g. possible to use a mono- or diacyloxyalkyl compound as R"'. A suitable pharmaceutical composition according to the invention is one as defined above wherein R'" comprises an acylgroup of 10-26 carbon atoms in length. For micelle formation a length of 10-15 will be suitable. For liposome formation longer chains are generally required in order to provide the desired hydrophobicity. Chain lengths of 12-24, even 14-24 are suitable. Preferably chain lengths exceed 14. Saturated or non saturated fatty acids can be used in both instances. Cis or trans fatty acids can be applied. As stated above this is common to liposome or micelle technology. With regard to chain lengths this counts also in general terms to R" and R"', not only for fatty acids as embodiment of R"'. Such chains can be alkyl, alkenyl, optionally substituted by amino, hydroxy and/or acyloxy or a mixture thereof. Such hydrophobic chains may comprise one or more fatty acid chains. These can vary in length or be of the same length. In a suitable embodiment naturally occurring fatty acids can be used. The use of natural fatty acids is preferred as tolerance to such a compound will be higher upon release of the active Pt, whereby the ligands are also released. The risk of side reactions will be lower using a component as ligand that occurs in vivo in the body e.g. as phospholipid membrane component. In general fatty acids with a chain length of 10-24 are suitable. A list of suitable chains is for example available in a general text book on fat constitution e.g. "The chemical constitution of natural fats" by T.P. Hilditch 2nd ed. 1949 page 6 and 7. The list of fatty acids listed there is hereby incorporated by reference. Other non natural derivatives are also suitable and will be apparent to a person skilled in the art. Oleoyl and myristoyl chains are particularly suited for example.

A pharmaceutical composition according to the invention wherein R"'= mono or diacylglyceryl forms a suitable embodiment of the invention. A glycerol derivative is an extremely suitable example of R"'. Alternatively a more complex alcohol derivative can be applied e.g. sphingosine. Other alternatives will be apparent to a person skilled in

the art. Shingosine is particularly suited as it forms lipids in nature.

A pharmaceutical composition according to the invention preferably comprises a phosphate group in the anionic organic compound. A particularly suitable embodiment comprises a phosphatide as R". Alternatively the anionic organic compound can comprise a sulphate group. Such is particularly suitable in the embodiment disclosed above with A and R"'. A pharmaceutical composition according to any of the preceding embodiments, wherein the anionic organic compound comprises a phospholipid forms a preferred embodiment of the invention. In particular a pharmaceutical composition according to any of the preceding embodiments, wherein the anionic organic compound is a phosphoglyceride is suitable. A good example of a compound according to the invention ccomprises as anionic organic compound a 1,2-diacyl-sn-glycero-3-phosphate.

Examples of the anionic organic complexant are amino acid residues (-OCO-CHR"-NR'-), malonic acid derivatives (-OCO-CHR"-COO-), α-phosphonocarboxylic acid derivatives (-OCO-CHR"-PO(O^-)-O-) and the corresponding amides.

A pharmaceutical composition wherein the anionic organic compound comprises a phosphorylated tyrosine, serine, homoserine or threonine structure is a preferred embodiment of the invention. The presence of the structures corresponding to other amino acids either natural or derived i.e. comprising an amino and carboxyl group also falls within the scope of the invention. A pharmaceutical composition according to the invention wherein the anionic organic compound is derived from serine (A = CH_2), forms a preferred embodiment. In particular in the embodiment disclosed above with A and R"', wherein the amino acids are comprised in the structure to the left of the phosphate group form suitable embodiments as does a pharmaceutical composition according to any of the preceding embodiments mutatis mutandis wherein A=CH2.

A pharmaceutical composition according to the invention preferably releases the active cytostatic compound upon contact with gluthathione. In general it is preferable that the complex is more stable in the bloodstream than in the cell.

A pharmaceutical composition according to the invention comprises the complex in a pharmaceutically acceptable dosage form. Such dosage forms are immediately apparent to a person skilled in the art. The dosage form will depend on the patient, the severity of the

malignancy, the dosage regime selected by the physician and can have numerous embodiments as is well known in the art. A number are provided here. A pharmaceutically acceptable dosage form can be selected from the group comprising tablets, lozenges etc, ingestible liquids, injectable solutions or freezedried products to be solubilised that can be administered via infusion i.a. The amount of active compound in a single bolus dosage form according to the invention can surpass that common to the prior art compositions comprising the corresponding cisplatinum derivative, due to the reduced toxicity of the subject compositions vis a vis the prior art compositions. A pharmaceutical composition must be sterile. It is preferably isotonic upon administration and should be free of undesirable compounds e.g. toxic compounds other than a cytostatic compound and impurities.

Two documents describe solutions of phosphatidylserine and cisplatin as aquated and/or chloride species. These documents do not deal however with production of a pharmaceutical composition according to the invention. In fact they do not disclose pharmaceutical compositions as such merely solutions with these two components. The solutions do not form compositions even inherently suitable as pharmaceutical compositions due to he presence of undesirable additives such as EGTA and absence of isotonic and sterile conditions.

The article by Taylor et al (IV) is directed at formation of complexes between these components in a gel like environment. It is stated that the interaction between DMPS and DDP seems to be very slow and difficult whereas cellular uptake of DDP seems to be quite fast. Also both DDP-OH and DDP-Cl appear to be bound. They indicate the phosphate group as being a binding partner. It is also suggested the aquated species binds to a greater extent than the Cl species due to interaction with the phosphate group. This suggests that DDP-OH would be stably bound to phosphatidyl serine in the cell. The cited article would thus seem to preclude application of such a complex for treatment requiring release of cisplatinumcompound in the cell. According to the cited article a person skilled in the art would assume DDP-OH level in the cell to be lower than DDP-Cl due to more stable binding. It also appears to tech that presence of a CP-PS complex of whatever type is not a likely event. Nothing is taught about any such complex being a suitable adminstration form of a cytostatic or suggests any advantages in this direction that could be used by a person skilled in the art faced with the problem of improved dosage forms of cisplatinum compounds.

The second article is written by the inventors (V) and describes the phenomenon that divalent ions electrostatically interact with various negatively charged membrane components i.a. phosphatidyl serine. They also disclose this phenomenon for phosphatidic acid, cardiolipin, phosphatidylinositol and phosphatidylglycerol. Nothing is taught or suggested about specific complex formation with cisplatin derivatives of any type certainly not of the type according to the invention with all the inherent advantages thereof resolving a long felt need.

A method of treating a subject having neoplasms with a compound of the cisplatinum family of cytostatic compounds said method comprising application of a pharmaceutical composition in any embodiment described above as the invention in a pharmaceutically acceptable dosage and form to said subject in order to reduce the number and/or size of neoplasms also falls within the scope of the invention. The neoplasms that can be treated are those which are already known in the prior art susceptible to treatment with cis platinum derivatives. Other forms may also be treated due to the higher dosages now possible in a more effective manner. A method according to the invention can reduce the number and/or size of neoplasms with reduced side effects related to treatment with said cytostatic compound were it to be applied as a direct release pharmaceutical composition per se. In particular a method according to the invention can reduce the number and/or size of neoplasms with reduced nephrotoxicity related to treatment with said cytostatic compound were it to be applied as a direct release pharmaceutical composition per se.

The invention also comprises use of a complex defined in the general formula of claim 1 in any of the embodiments of the subsequent claims as active component for preparing a pharmaceutical composition suitable for treating a subject having neoplasms with a view to reducing size and/or number of neoplasms, said pharmaceutical composition exhibiting reduced side effects in comparison to the cytostatic compound provided by said complex were said cytostatic compound to be applied as a direct release composition per se.

The following examples elucidate the invention but are not considered to restrict the scope of the invention. The content of the listed references is deemed to be incorporated by reference.

EXAMPLE

Cisplatin complexes with phosphatidylserine in membranes*

INTRODUCTION

cis-Diamminedichloroplatinum(II) (cisplatin)[1] is a commonly used anti-cancer drug. It is a reactive compound which can occur in various species in aqueous solution depending on the pH and chloride ion concentration (1-4). It is well established that cisplatin interacts with DNA and thereby causes inhibition of DNA synthesis which might be a primary therapeutic action (5-7). In addition the molecule interacts with proteins (8, 9). Whether cisplatin has an affinity for cellular lipids is not known. Knowledge about cisplatin-lipid interactions might contribute to a better understanding of the antitumor activity of cisplatin but might also shed light on the severe side-effects of the drug of which nephrotoxicity is the most serious (4, 10, 11). A relationship between nephrotoxicity and interaction of positively charged aminoglucosides with negatively charged phospholipids (12, 13) has been suggested.

We recently reported the binding of cisplatin to model membranes of varying phospholipid composition (14). It was observed that the positively charged aquated species of cisplatin binds to different anionic lipids which can result in changes in lipid packing. In the course of these investigations it was noticed that cisplatin specifically shows a strong interaction with phosphatidylserine (PS) (14). Also in another recent study a binding of cisplatin to PS was observed (15). In the present study the formation of a stable complex with PS is described. It is shown that both in model and in biological membranes cisplatin coordinatess specifically with PS involving a loss of two chloride ions (or in case of aquated cisPt two water molecules) and coordination of platinum to the amine and carboxylate group of the serine moiety. Because PS is involved in many cellular functions such as transmembrane signaling (16, 17) this implies that cisplatin-PS complex formation quite likely interferes with these functions.

We also determined that toxicity of liposomal platinum according to the invention was less than for an equivalent dose of conventional platinum notwithstanding the greater availability of free platinum upon liposomal application as determined in pharmacokinetic experiments.

EXPERIMENTAL PROCEDURES

Materials - Cisplatin was purchased from Sigma (St. Louis, MO, USA). Phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL, USA) and contained two oleoyl fatty acyl chains, except cardiolipin, derived from bovine heart, sphingomyelin, derived from egg, and phosphatidylinositol, derived from soybean.

Cisplatin incubations with phospholipids dispersions - Lipid dispersions (2 mM phospholipid) were prepared by adding buffer containing no or 5 mM cisplatin to a dry lipid film followed by 10 times freeze-thawing and agitation with a Vortex mixer. In all cases cisplatin solutions were always freshly prepared by dissolving the compound in the appropriate buffer which was facilitated by warming to 40-50 °C. Buffers employed were 10 mM Pipes, 50 mM Na_2SO_4, 1 mM egta, pH 7.4; 10 mM Mes, 50 mM Na_2SO_4, 1 mM egta, pH 6.0; 10 mM Pipes, 100 mM NaCl, 1 mM egta, pH 7.4; and 10 mM Mes, 100 mM NaCl, 1 mM egta, pH 6.0. The samples were incubated in the dark at room temperature for the indicated times after which the lipids were extracted (18) and analyzed by TLC on silicagel eluting with chloroform/methanol/acetic acid (7/2/1, v/v), chloroform /methanol /ammonium/water (34/14/1/1, v/v), or chloroform/methanol/water (35/13/2 v/v). Lipids were identified by spraying with either 10% H_2SO_4 followed by charring, phosphorus or ninhydrin spray reagents. For quantitative analysis lipid spots were scrapped off after which the material was destructed by perchloric acid and the amount of phosphorus was quantified (19).

Cisplatin incubations with red blood cells ghost - Human red blood cells ghost isolated according to (20). 5 mM cisplatin (final concentration) was added to the membranes (2 mM phospholipid-Pi) and incubation was performed at 37 °C, in 10 mM Mes, 50 mM Na2SO4, 1 mM egta, pH 6.0. Since these ghosts are open structures, cisplatin is able to reach both the inner and outer leaflet of the membrane. Samples were taken at different time points and phospholipids were extracted according to (18). TLC experiments were performed in 1 dimension with chloroform/methanol/acetic acid (7/2/1, v/v) as eluens and sprayed with ninhydrin or in 2 dimensions with in the first dimension chloroform/methanol/ammonium/water (34/14/1/1, v/v) and in the second dimension chloroform/methanol/acetic acid (7/2/1, v/v) after which lipids

were identified by spraying with phosphorus spray reagent.

Mass spectrometry - Cisplatin was incubated with 1,2-dioleoyl-*sn*-glycero-phosphoserine (DOPS) as described above after which the lipids were extracted (18). The chloroform phase was used to analyze the product. Positive FAB mass spectra and CID MS-MS spectra were obtained using a Jeol JMS-SX102/102A four sector instrument. For the FAB mass spectra NBA was used as a matrix. The MS-MS spectra were acquired with sufficient resolution of MS1 using nitrogen as collision gas. The gun was operated at 6 kV ad at 5 mA current. The sample was dissolved in $CHCl_3/CH_3OH$ and added to the matrix. The mass spectrum shows a cluster of protonated molecular ions at m/z 1014, 1015, and 1016. The intensity ratio and the mass value are in agreement with the elemental formula of the proposed Pt complexes. From the ions mentioned above, representing the most abundant Pt isotopes, MS-MS spectra were recorded.

NMR ∘ spectroscopy- Cisplatin was incubated with DOPS after which the lipids were extracted as described above. ^{1}H, ^{13}C, ^{31}P, and ^{195}Pt NMR spectra were recorded in saturated solutions of $CDCl_3$ (^{1}H, ^{13}C, and ^{31}P) and in $CDCl_3$/MeOH 9:1 (^{13}C, ^{31}P and ^{195}Pt); spectra were referenced to TMS, TMP (trimethyl phosphate) and K_2PtCl_4 (-1600 ppm), respectively.

In vivo tests

1) Toxicity testing in mice was carried out using conventional platinum and the compound according to the invention (cis Pt-DOPS complex) in increasing dosage and a standard volume of physiological salt. Mortality and morbidity were analyzed.

2) Pharmacokinetics were analyzed on the basis of blood samples taken from mice at various time intervals after a single injection with either the compound according to the invention i.e. Cis Pt-DOPS complex or conventional platinum. The AUC from the resulting curves was determined using the trapezoidal method. The curves were fit to a 2-compartment system.

RESULTS AND DISCUSSION

Cisplatin specifically complexes with phosphatidylserine in model membranes - When DOPS is dispersed in a chloride-ion free buffer of

pH 6.0 (10 mM MES, 50 mM Na_2SO_4, 1 mM egta) containing 5 mM cisplatin and incubated at room temperature for 3 days a new species (X) can be observed by TLC (Fig. 1A). X has a lower R_F value then DOPS in an acidic eluens, it is phosphorus positive and in contrast to DOPS it does not stain purple (but brownish) when stained with the amino-group-specific ninhydrin reagent (data not shown). Dispersions of DOPE, DOPC and sphingomyelin, representatives of the other major mammalian plasma membrane phospholipids, do not show the appearance of new reaction products upon incubation with cisplatin under these conditions (Fig. 1A). This is also not observed for a DOPE/DOPC (1:1, molar) mixed dispersion (data not shown) which is organized in a lamellar phase at room temperature (pure DOPE forms a H_{II} phase [21]). Furthermore, new reaction products are also absent when the negatively charged phospholipids cardiolipin, phosphatidylglycerol and phosphatidylinositol are incubated with cisplatin (data not shown).

Formation of X at room temperature is a slow process with an estimated half-time of 70 hours (Fig. 1B) and is paralleled by a reduction in free DOPS suggesting that X is the product of the DOPS - cisplatin interaction.

The most reactive species of cisplatin is not the native drug but the positively charged, aquated species (4). When cisplatin becomes in contact with water, chloride ions are stepwise replaced by water molecules resulting in mono- $[(NH_3)_2PtCl(H_2O)^+]$ and diaquated $[(NH_3)_2Pt(H_2O)_2^{2+}]$ species. This process is inhibited by Cl^-. The mono- and diaquated species are weak acids with pKa's of 6.41 and 5.37, respectively (1, 2). To test which species is responsible for the reaction with DOPS the lipid was dispersed and incubated at 37 °C in buffers with no or 100 mM NaCl and at pH values of 6.0 and 7.4. Because the properties of anionic lipids like PS depend on the nature and amount of cations in the buffer the chloride free buffer was supplemented with 50 mM Na_2SO_4. After certain time intervals the samples were analyzed by TLC and the percentage of conversion of PS was quantified (Fig. 2). Comparing the incubation of $[Cl^-]$ = 0, pH 6.0 in Fig. 1B (room temperature incubation) with that in Fig. 2 immediately reveals that at the clinically relevant temperature of 37 °C the conversion of PS is greatly increased, with a half-time of the reaction of 5 hours. In the absence of Cl^- the reaction rate is slightly lower at the higher pH value. Furthermore it is clear from Fig. 2 that also in the Cl^- containing buffers the reaction occurs to a significant extent, but at an

20- to 30-fold reduced rate for the two pH values tested.

Structure of the cisplatin-phosphatidylserine complex - The lower R_F value in an acidic eluens (Fig. 1A) and higher R_F in an alkalic or neutral eluens (data not shown) indicate that the complex is less negatively charged than DOPS and probably neutral. In order to examine the structure of the cisPt - DOPS complex mass spectrometry was performed. The mass spectrum shows a $(M+H)^+$ cluster around m/z 1015, (Fig. 4). Based on the intensity ratio and the m/z values of the ions in this cluster the molecule must contain platinum. A calculated isotope distribution of a molecule containing 42 C, 83 H, 3 N, 10 O, 1 P, and 1 Pt is inserted in Fig. 4.

The $(M+H)^+$ ions at m/z 1014, 1015, and 1016 were subjected to MS-MS. In the MS-MS spectra two dominant fragments ion clusters are observed m/z 412, 413, and 414, and m/z 314, 315, and 316 (Fig. 5). In the product ion spectrum of m/z 1014 only m/z 412 and m/z 314 are observed (Fig. 5A). In the MS-MS spectrum of m/z 1015 the peak intensity ratios I(m/z 412)/I(m/z 413) and I(m/z 314)/I(m/z 315) correspond to the ratio between the ^{13}C and ^{195}Pt contribution (35:65) in the selected precursor ion (Fig. 5B). Three major components contribute to the m/z 1016 ion, namely: $(^{13}C)_2{}^{194}Pt$, $^{13}C^{195}Pt$, and ^{196}Pt in an intensity ratio of approximately 10:35:55. In the product ion spectrum of m/z 1016 we observed again two fragment ion clusters each containing 3 peaks with relative intensities corresponding to the component distribution in the precursor ion. These observations allow to conclude that the fragment ions mentioned above all contain a platinum atom.

The existence of these fragments can only be explained if the cisPt - PS complex has a structure as shown in Fig. 6A, in which the platinum is coordinated to the carboxylate and amine group of the serine moiety. This molecule has a nominal molecular weight of 1015 dalton in its protonated form, and upon fragmentation of this complex by MS-MS platinum containing fragment ions of m/z 412 and m/z 314 dalton are obtained. The former fragment indicates that platinum is not associated with the fatty acyl chain part or the glycerol backbone but with the headgroup (Fig. 6B). The latter fragment can be explained by a loss of H_3PO_4 from the serine moiety (Fig. 6C). The structure shown in Fig. 6A is at a physiologically relevant pH zwitterionic.

From ^{31}P spectroscopy a shift observed at 0.67 agrees with phosphate groups not coordinated to platinum. The signal observed in the

^{195}Pt spectrum at -2159 ppm is in excellent agreement with a chromophore [PtN_3O]; although theoretically also unreacted cisplatin could be in this range. As a reaction has occurred because the ^{13}C signal at 184.6 ppm agrees with platinated carboxylate group, it can be concluded that all NMR data agree with the structure proposed in Fig. 6A.

Interaction of cisplatin with phosphatidylserine in erythrocyte membranes - Subsequently, it was examined whether the formation of cisplatin-phosphatidylserine coordination complex also occurs in biological membranes, where mixtures of phospholipids and proteins are present. Human erythrocyte ghosts were used to study the interaction of cisplatin. A two-dimensional TLC was performed in order to get a good separation of the phospholipid mixture. Fig. 7A demonstrates that in the presence of cisplatin the cisplatin-PS product appears next to the PS spot with a higher R_F value in the first dimension (alkalic eluens) and a lower R_F value in the secon dimension (acid eluens). From Fig. 7B it is clear that after incubation with cisplatin the amount of PS is significantly reduced in a time-dependent manner, whereas the amount of phosphatidylethanolamine remains constant. Furthermore, in control experiments without cisplatin present during incubation the amount of PS remains constant in time. Therefore, the interaction of cisplatin with PS not only takes place in model membranes, but also in biological membranes.

PS is exclusively located in the inner leaflet of the plasma membrane and contributes 20 % to the phospholipids present in this leaflet (22). At this side of the membrane the pH and Cl^- concentration are lower than that of the extracellular compartment, i.e. the highest concentration of aquated, reactive cisplatin is formed at the side were PS is located. PS plays a crucial role in cellular processes such as signal transduction (16), cell proliferation and apoptosis (23), and the blood clotting cascade (24) the biogenesis of mitochondria (275, and is necessary for the activity of enzymes such as Na^+/K^+-ATPase (26) and protein kinase C (16). For the latter enzyme the carboxyl acid moiety of PS is involved (16, 17), which is now obscured by the interaction with cisplatin. Indeed, effects of cisplatin on the activity of this enzyme have been reported (28, 29). It is therefore highly interesting to study the effect of the cisplatin -PS interaction on PS-dependent processes, and its consequences for the mechanism of cisplatin toxicity and side-effects, such as nephrotoxicity.

The results from the in vivo tests were as follows:

1) In vivo toxicity. A single injection of physiological salt by way of control was as expected non-toxic. The dose levels were 1, 5 and 10 mg/kg. The lowest dose level was tolerated well for both compounds. (n=8/group). There was no mortality, no morbidity and weight increase was normal. The results for 5 mg/kg were comparable. At dose level 10 mg 6/6 mice died within a week of treatment with conventional platinum. In contrast the treatment group with the compound according to the invention showed no mortality and reasonable tolerance. After 7 days this group exhibited morbidity in the shape of general malaise and raised hair.

2) Pharmacokinetic test showed the following results

	Free			Total		
	$t_{1/2}(1)$ (h)	$t_{1/2}(2)$ (h)	AUC 0-48H (hμM)	$t_{1/2}(1)$ (h)	$t_{1/2}(2)$ (h)	AUC 0-48 h (hμM)
cis	1.06	107.89	3.86	3.21	41.02	48.10
cis-lip	2.08	25.91	8.70	2.54	21.71	50.52

The AUC for the compound according to the invention (free) is higher than the AUC for the conventional drug (free). This indicates a larger availability of cisplatin upon release from the liposome. The AUC for the total value was approximately equivalent.

Interaction of Cisplatin with Phosphatidylserine Can Be Reversed with Glutathione.

To examine whether the complex formed between cisplatin and DOPS can be reversed, experiments were performed in which this complex was incubated with glutathione. In Figure 3 it is shown that a concentration- and time-dependent disappearance of the complex and concomitant reappearance of DOPS occurs upon incubation with glutathione. This phenomenon was observed even under conditions of high [CI^-] and pH 7.4 (Figure 3). Glutathione was chosen because it is known to compete for cisplatin complexation with other (macro)molecules in the cell, such as DNA. Intracellular glutathione therefore plays an important role in the anticancer activity of cisplatin (Timmer-Bosscha et al., 1992 [Document V]). Furthermore, cisplatin-glutathione complexes can be extruded by (cancer) cells by a GS-X pump (Ishakawa et al., 1994 [Document VI]),

thereby also decreasing the anticancer activity of the drug.

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1 The following abbreviations were used:

CID	Collision induced dissociation
cisplatin	*cis*-diamminedichloroplatinum(II)
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-*sn*-glycero-3-phosphoserine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
egta	ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FAB	Fast Atom Bombardment
Mes	2-morpholinoethanesulphonic acid
MS	mass spectrometry
MS-MS	tandem mass spectrometry
NBA	methyl-Nitrobenzylalcohol
Pipes	piperazine-1,4-diethanesulfonic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
TLC	thin layer chromatography

LEGENDS TO THE FIGURES

Fig. 1: Formation of a product of DOPS upon incubation with cisplatin. (A) Thin layer chromatography of DOPS, DOPE, DOPC and sphingomyelin. LUVET composed of these lipids were incubated in buffer composed of 10 mM Mes, 50 mM Na_2SO_4, 1 mM egta, pH 6.0 for 72 h at room temperature in the presence or absence of 5 mM cisplatin. Lipids were extracted and subjected to TLC with as eluens chloroform/methanol/acetic acid (7/2/1 v/v) and visualized with phosphorous spray. (B) Conversion of DOPS in time. DOPS was incubated with 5 mM cisplatin at room temperature in the buffer described above. Samples were taken and extracted and subjected to TLC. The increase in the DOPS - cisplatin reaction product was based on the phosphor content of the product spot on the TLC plate. 100 % is the sum of the amount of phosphor in the product spot and in the DOPS spot .

Fig. 2: Conversion of DOPS at 37 °C in buffers of various composition. DOPS was incubated in the presence of 5 mM cisplatin. (A=●) Incubation in buffer composed of 10 mM Pipes, 50 mM Na_2SO_4, 1 mM egta, pH 7.4. (B=○) Incubation in buffer composed of 50 mM Mes, 50 mM Na_2SO_4, 1 mM egta, pH 6.0. (C=■) Incubation in buffer composed of 10 mM Pipes, 100 mM NaCl, 1 mM egta, pH 7.4, and (D=□) Incubation in buffer composed of 10 mM Mes, 100 mM NaCl, 1 mM egta, pH 6.0.

Figure 3: Conversion of the cisplatin-DOPS complex at 37°C by glutathione, DOPS/DOPC lipid dispersions (1/1) were incubated overnight in the presence of 5 mM cisplatin in 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0, at 37°C. Lipids were washed and resuspended at 10 mM Pipes, 100 mM NaCl, and 1 mM EGTA, pH 7.4, containing either 0 (●), 15 (○), or 100 mM (■) glutathione, DOPS and cisplatin-DOPS were analyzed as described under Experimental Procedures.

Fig. 4: Mass spectrometry of the product formed upon incubation of DOPS with cisplatin. The inset shows a calculation of the isotope distribution as can be expected for a molecule with a bruto formula of 42 C, 83 H, 3 N, 10 O, 1 P, and 1 Pt.

Fig. 5: MS-MS spectrometry of fragment ions obtained from the ion of m/z 1014 (A), m/z 1015 (B), and m/z 1016 (C).

Fig. 6: Proposed structure of the product formed upon incubation of DOPS with cisplatin with a molecular mass of 1015 dalton (A). Proposed structure of the large fragment ion obtained from MS-MS (B). Proposed structure of the small fragment obtained by MS-MS (C).

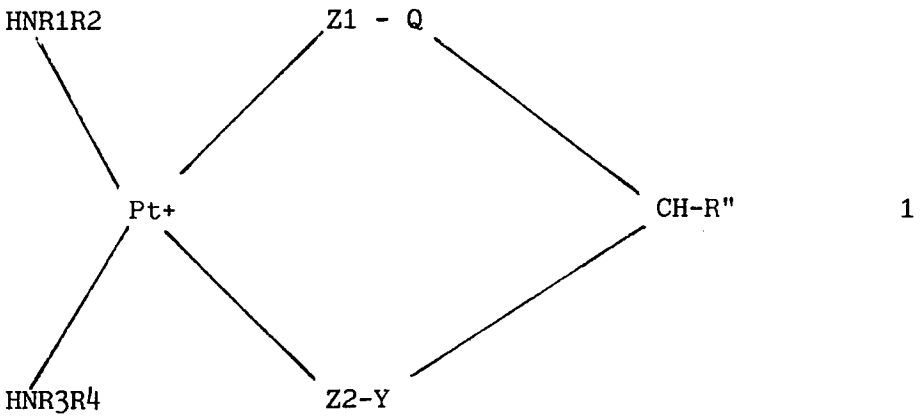
Fig. 7: Formation of a cisplatin-PS complex in biological membranes. Ghosts of red blood cells were incubated with or without cisplatin as described in the legend of fig. 1. (A) Lipids were analyzed with 2-dimensional TLC with in the first dimension chloroform/methanol/-ammonium/water (34/14/1/1, v/v) and in the second dimension chloroform/methanol/acetic acid (7/2/1, v/v) after which lipids were identified by spraying with phosphorus spray reagent. The upper panel shows lipids after incubation without cisplatin and the lower panel shows the incubation in the presence of cisplatin. (B) Lipids were analyzed after incubation with cisplatin for various periods of time, and

extraction with 1-dimensional TLC with chloroform/methanol/acetic acid (7/2/1, v/v) as eluens after which the plate was sprayed with ninhydrin.

Figure 8a and 8b: show the in vivo results of the pharmacokinetic experiments.

CLAIMS

1. A pharmaceutical composition for use in treatment of a subject having neoplasms with a view to reducing size and/or number of neoplasms, said pharmaceutical composition comprising a complex of a cytostatic compound of the cis platinum family and an anionic organic compound as a complexed form of the active pharmaceutical component, said complex having the following general formula 1



1

wherein

Q = -CO- or -PO(O^-)-;

Y = -CHR'-, -CO-, -PO(O^-)- or

a direct bond;

Z1, Z2 = independently O or NR', R1,R2=H or a hydrocarbon group optionally substituted, optionally linked to each other or to R3 and/or R4

wherein R3, R4=H or hydrocarbon group optionally substituted, optionally linked to each other or to R1 and/or R2,

wherein

R'=H, or a hydrocarbon group optionally substituted and wherein R"= hydrocarbon group substituted by a group capable of liposome formation.

2. A pharmaceutical composition according to claim 1, wherein the anionic organic compound comprises a phosphate group or a sulphate group, preferably a phosphate group.

3. A pharmaceutical composition according to claim 1 or 2, wherein

R" = -A-O-PO(O^-)-O-R"' or -A-O-SO_2-O-R"'
wherein A=CnH2n, n=1-4 optionally substituted e.g. with hydroxy, carboxy or phenyl, R' is as defined in previous claims,
R"'= alkyl or alkenyl, optionally substituted by amino, hydroxy, acylamino and/or acyloxy.

4. A pharmaceutical composition according to claim 3 wherein A = CH_2.

5. A pharmaceutical composition according to claim 3 or 4, wherein Z^1 = O, Z^2 = NH, Q = CO and Y = a direct bond.

6. A pharmaceutical composition according to any of claims 1-4 wherein R"'= triacylglyceryl.

7. A pharmaceutical composition according to any of the preceding claims, wherein the anionic organic compound is a phospholipid.

8. A pharmaceutical composition according to any of the preceding claims, wherein the anionic organic compound is a phosphoglyceride.

9. A pharmaceutical composition according to any of the preceding claims, wherein the anionic organic compound is a 1,2-diacyl-sn-glycero-3-phosphate.

10. A pharmaceutical composition according to any of the preceding claims, wherein R" comprises a group containing 10-24 carbon atoms.

11. A pharmaceutical composition according to any of the preceding claims, wherein R" comprises a group with a length and structure conducive to micelle formation e.g. of 10-15 carbon atoms in length.

12. A pharmaceutical composition according to any of claims 1-10, wherein R" comprises a group with a length and structure conducive to liposome formation e.g.of 12-24, preferably 15-24 carbon atoms in length.

13. A pharmaceutical composition according to any of claims 10-12, wherein R" comprises an acylgroup, e.g. oleoyl.

14. A pharmaceutical composition according to any of the preceding

claims wherein the cytostatic compound is a cis platinum compound selected from first and second generation compounds.

15. A pharmaceutical composition according to any of the preceding claims wherein the cytostatic compound is a cis diammineplatinum(II) derivative i.e. R1 = R2 = R3 = R4 = H.

16. A pharmaceutical composition according to any of the preceding claims whereby contact with gluthathione releases the active cytostatic compound.

17. A pharmaceutical composition according to any of the preceding claims wherein the complex is comprised in a pharmaceutically acceptable dosage form.

18. A pharmaceutical composition according to any of the preceding claims which is sterile.

19. A pharmaceutical composition according to any of the preceding claims which comprises the complex as a multilamellar or monolamellar vesicle or as a micelle.

20. A pharmaceutical composition according to any of the preceding claims which comprises the complex as a slow release form of the cisplatinum compound.

21. A method of treating a subject having neoplasms with a compound of the cisplatinum family of cytostatic compounds said method comprising application of a pharmaceutical composition according to any of the preceding claims in a pharmaceutically acceptable dosage and form to said subject in order to reduce the number and/or size of neoplasms.

22. A method of treating a subject having neoplasms with a compound of the cisplatinum family of cytostatic compounds said method comprising application of a pharmaceutical composition according to any of claims 1-20 in a pharmaceutically acceptable dosage and form to said subject in order to reduce the number and/or size of neoplasms with reduced side effects related to treatment with said cytostatic compound were it to be applied as a direct release pharmaceutical composition per se.

23. A method of treating a subject having neoplasms with a compound of the cisplatinum family of cytostatic compounds said method comprising application of a pharmaceutical composition according to any of claims 1-20 in a pharmaceutically acceptable dosage and form to said subject in order to reduce the number and/or size of neoplasms with reduced nephrotoxicity related to treatment with said cytostatic compound were it to be applied as a direct release pharmaceutical composition per se.

24. Use of a complex as defined in any of claims 1-20 as active component for preparing a pharmaceutical composition suitable for treating a subject having neoplasms with a view to reducing size and/or number of neoplasms, said pharmaceutical composition exhibiting reduced side effects in comparison to the cytostatic compound provided by said complex were said cytostatic compound to be applied as a direct release composition per se.

Fig.1a

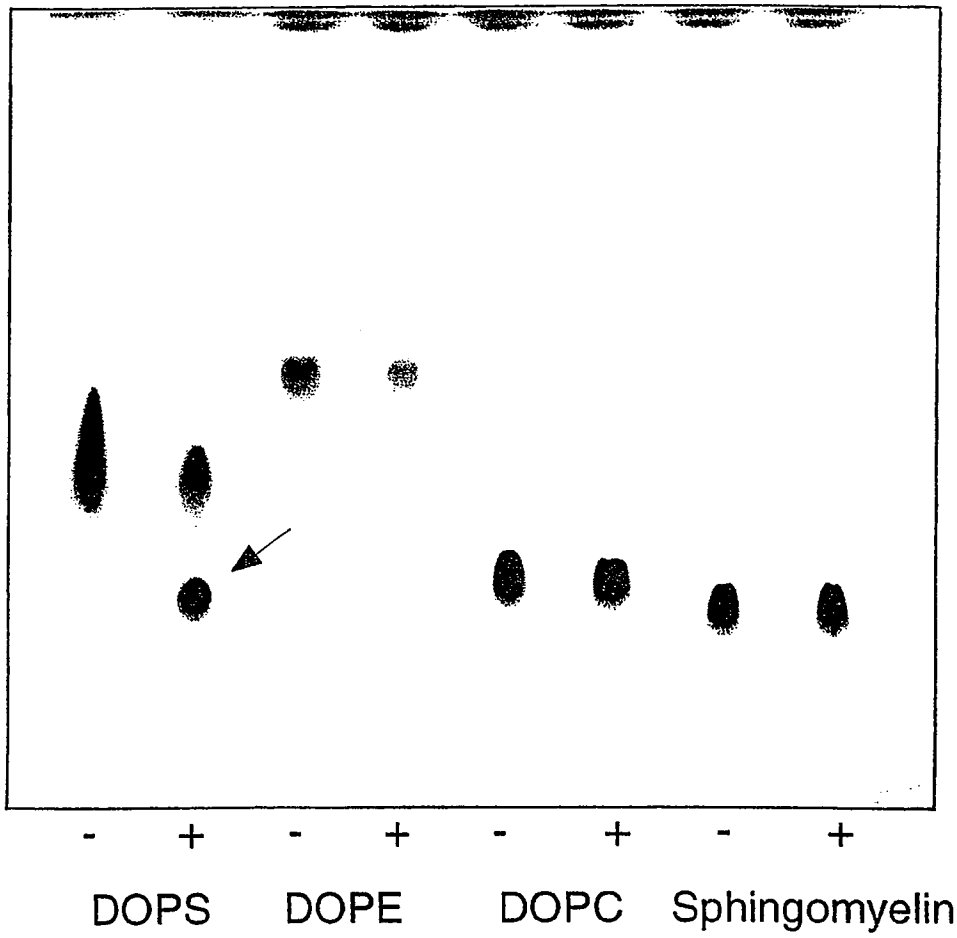

- + - + - + - +
DOPS DOPE DOPC Sphingomyelin

Fig.1b

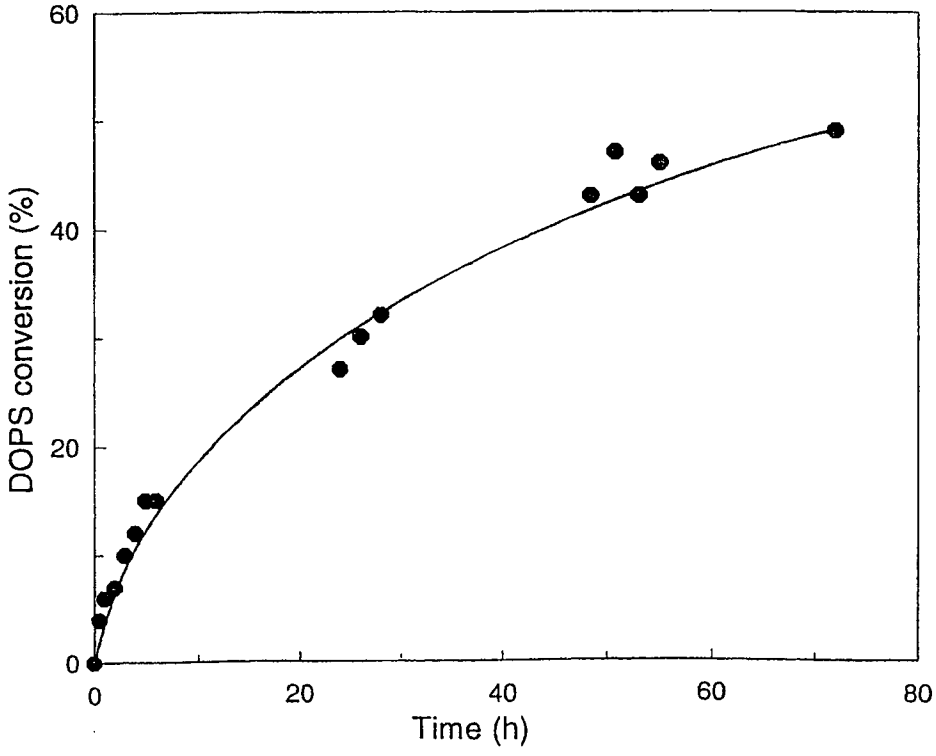

DOPS conversion (%)
0
20
40
60
0
20
40
60
80
Time (h)

Fig. 2

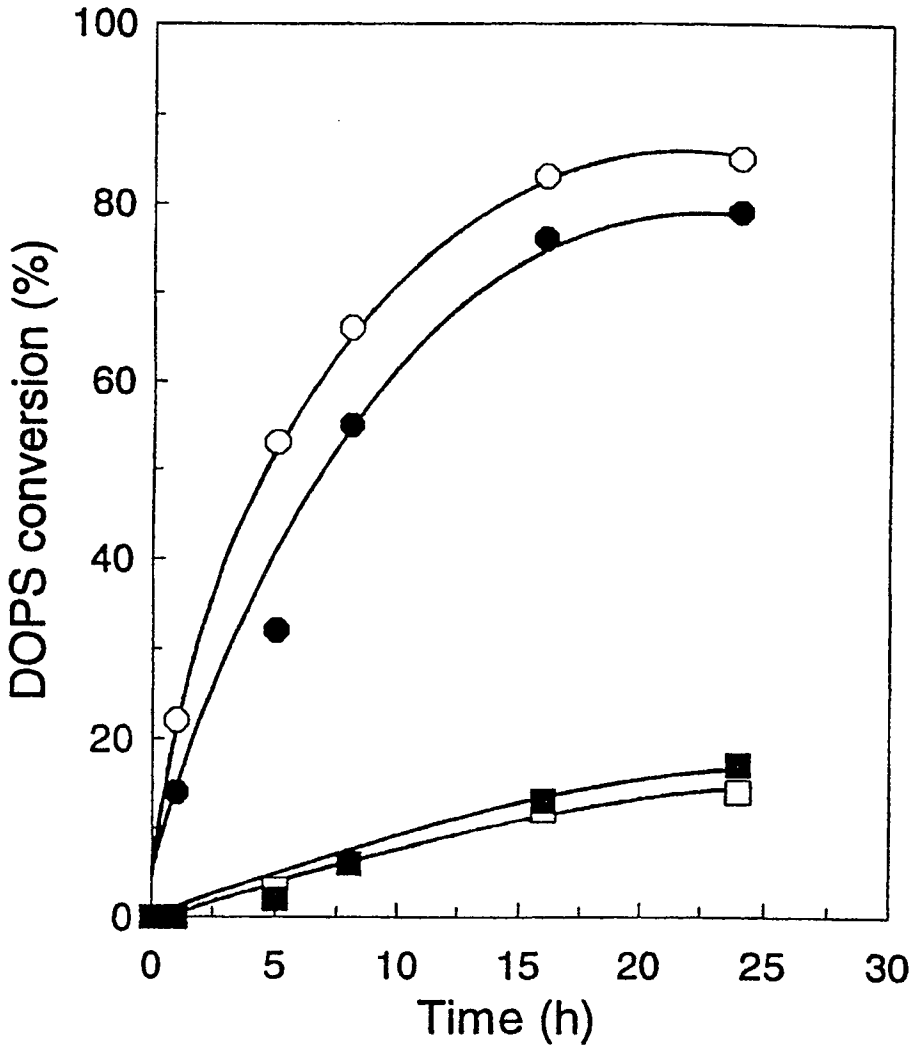


Fig. 3

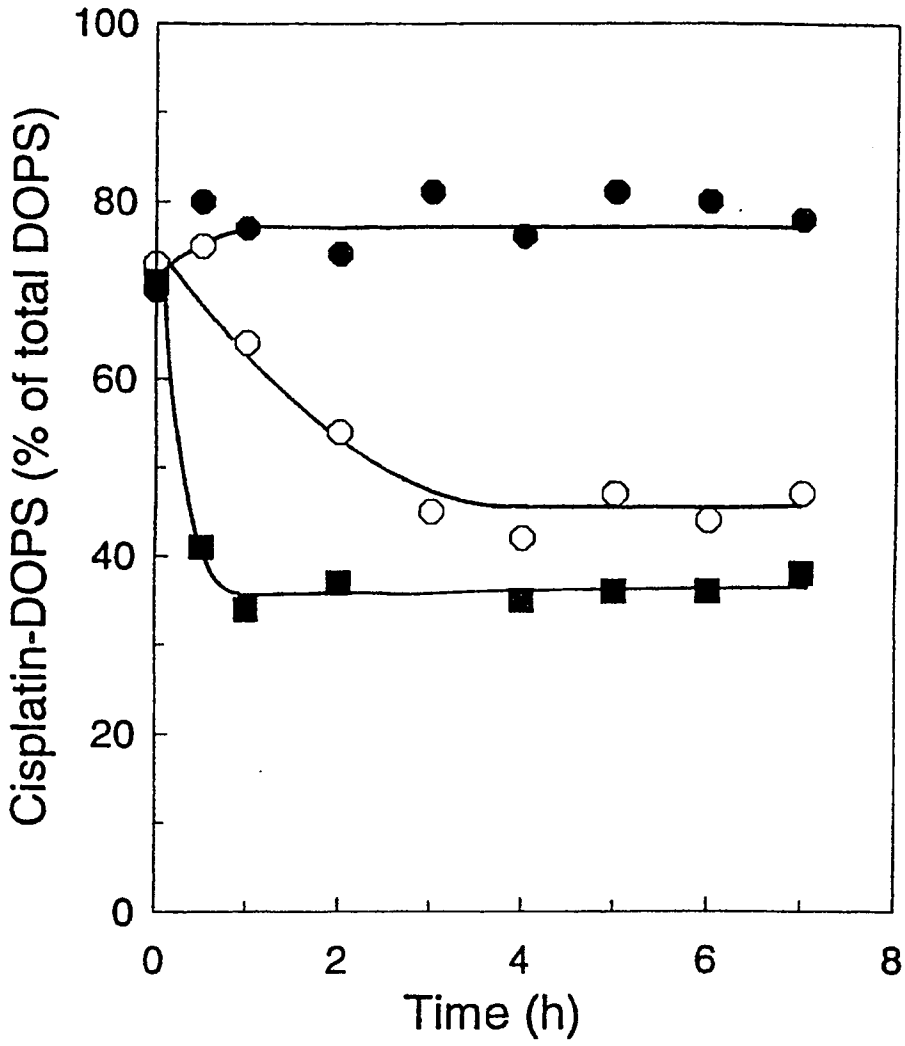


Fig. 4

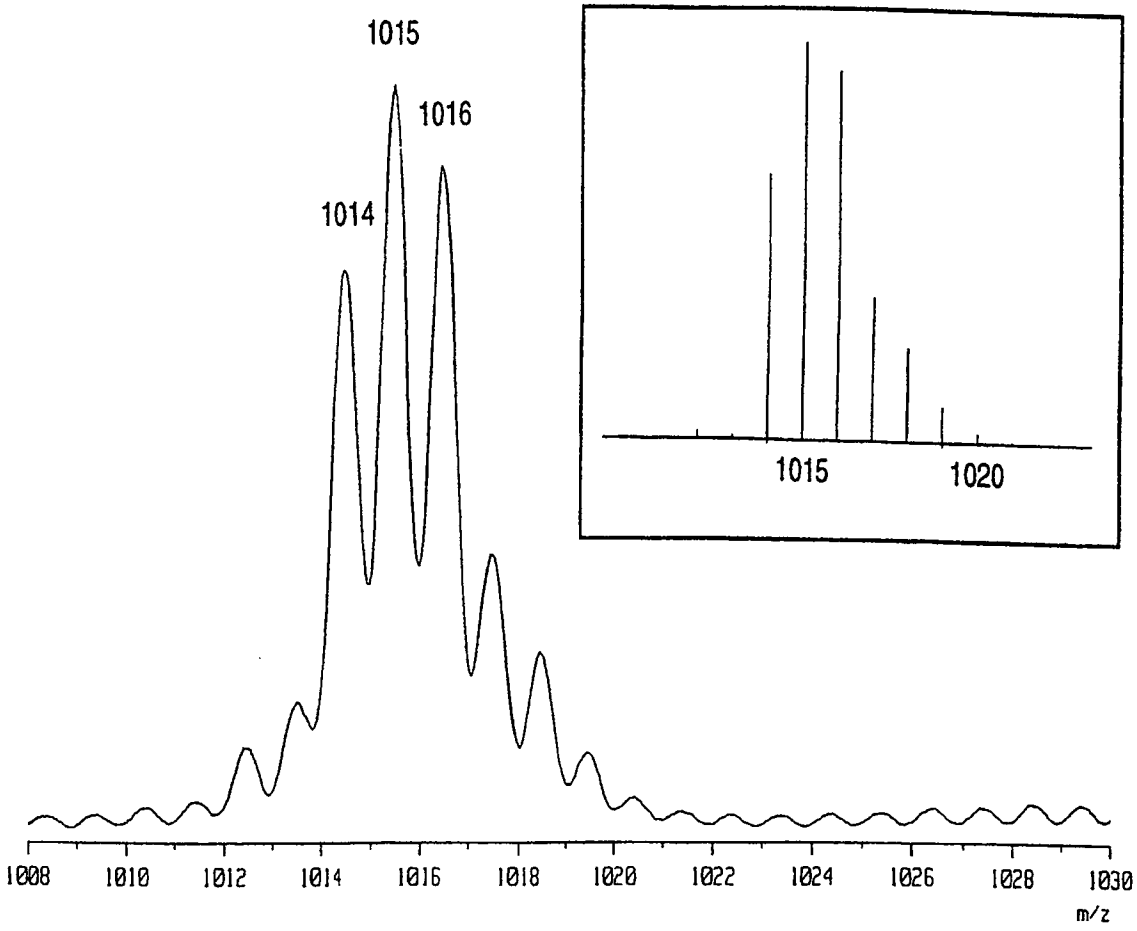

1015
1016
1014
1015
1020
1008
1010
1012
1014
1016
1018
1020
1022
1024
1026
1028
1030
m/z

Fig. 5a

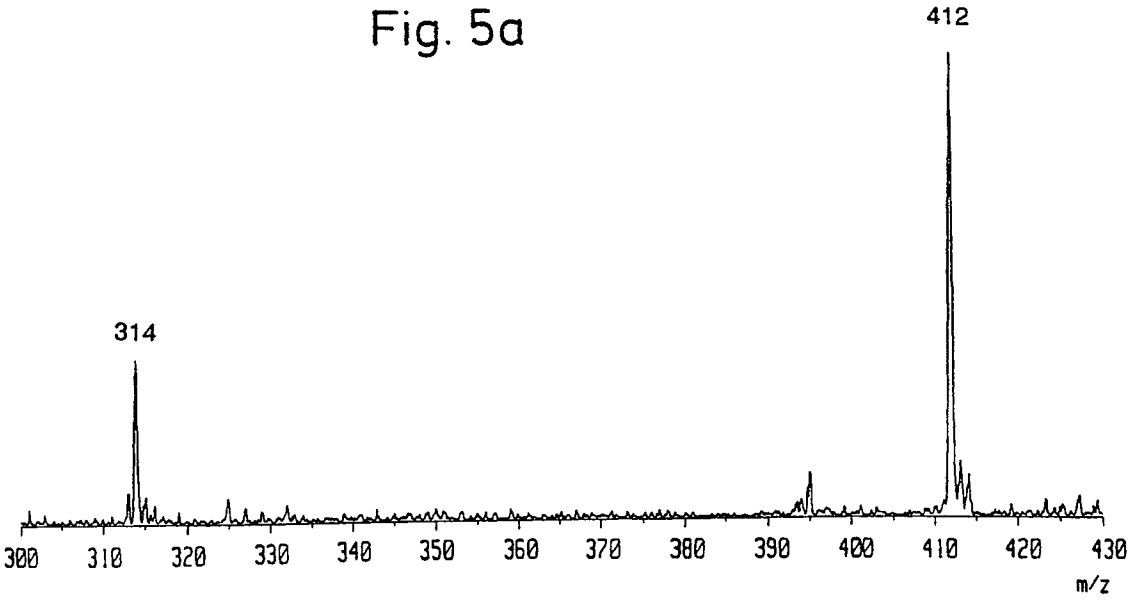


Fig. 5b

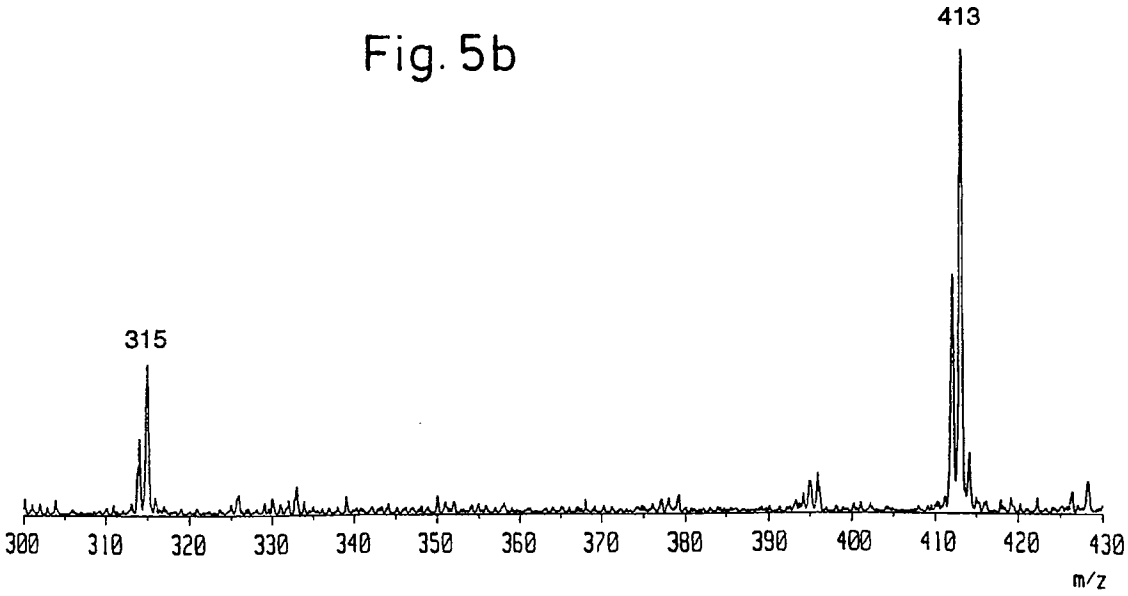


Fig. 5c

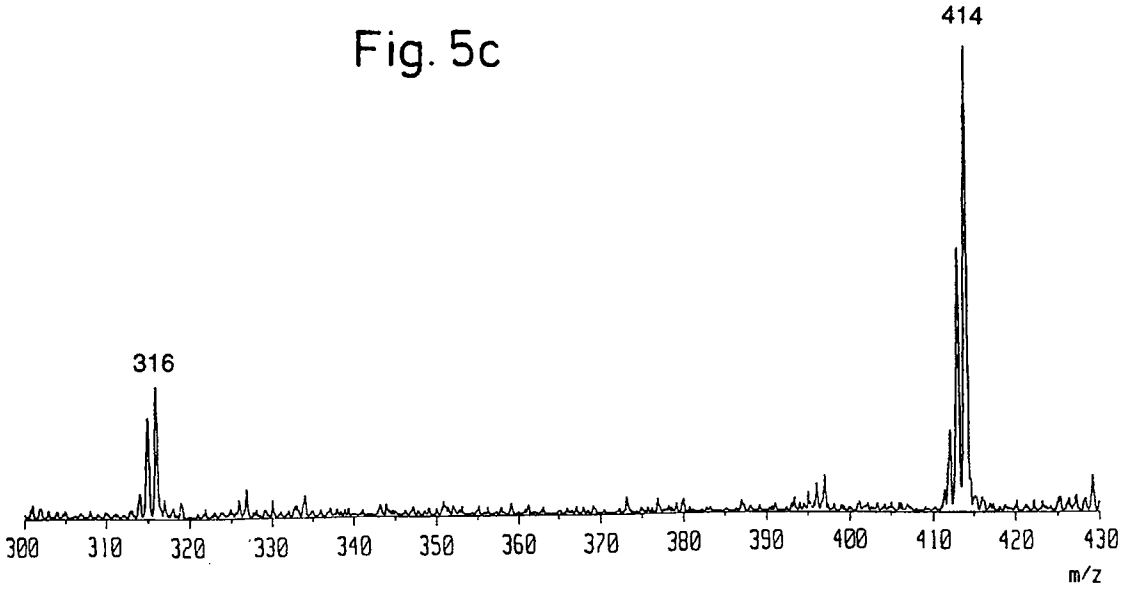


Fig. 6a

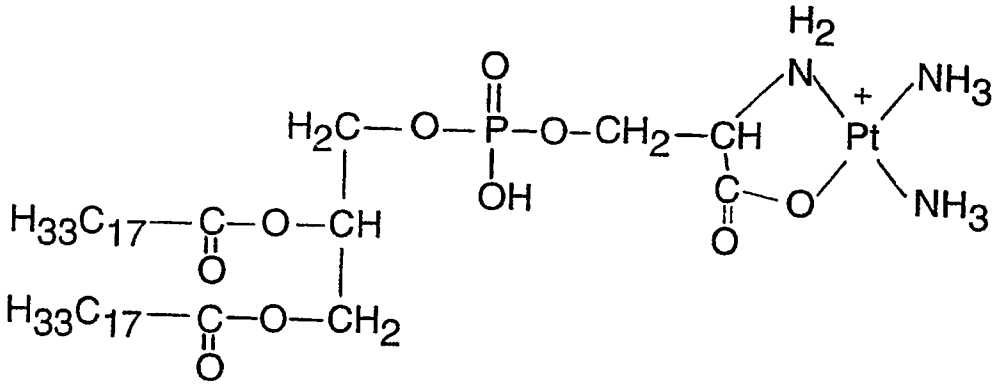


Fig. 6b

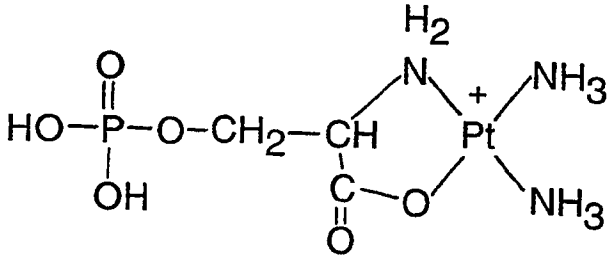


Fig. 6c

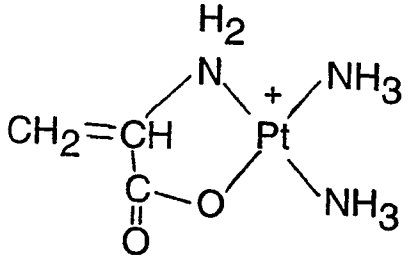


Fig. 7

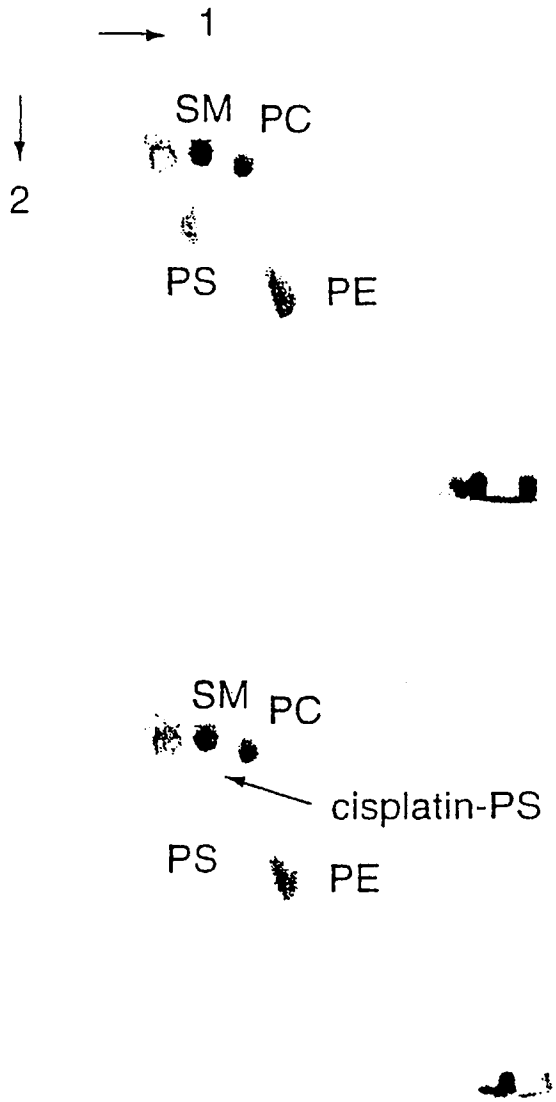


Fig. 8a

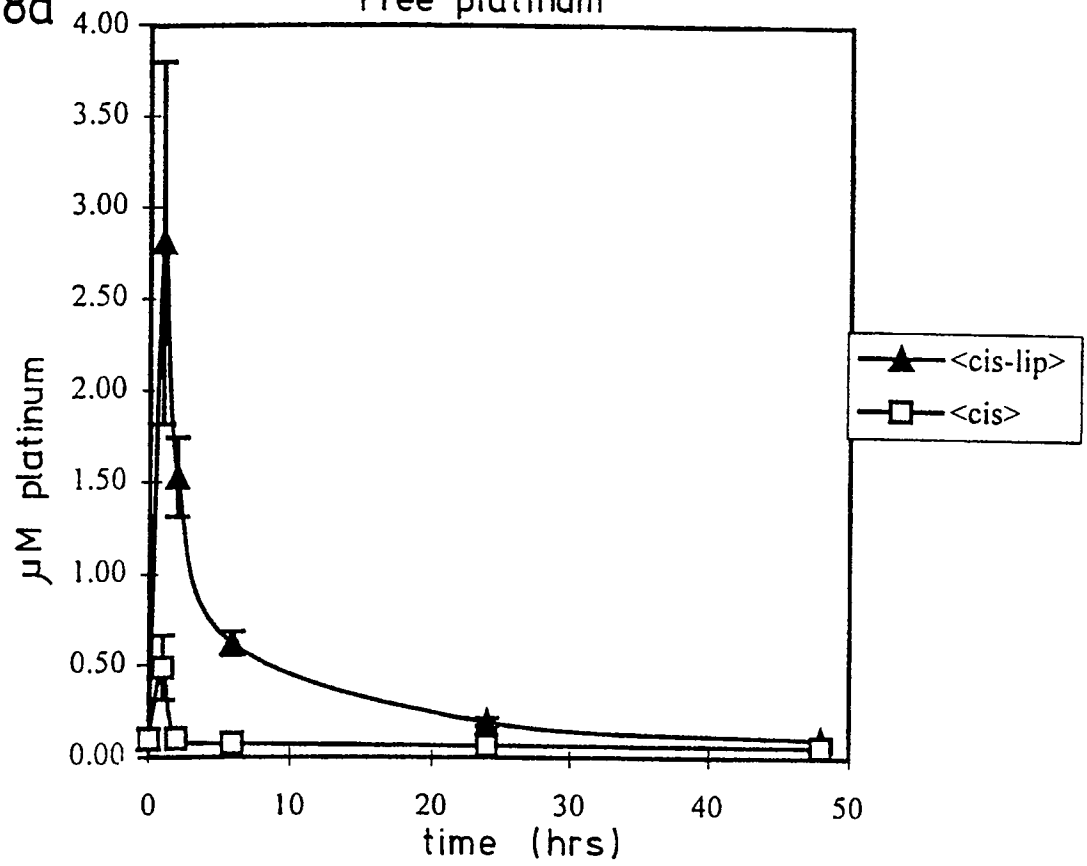
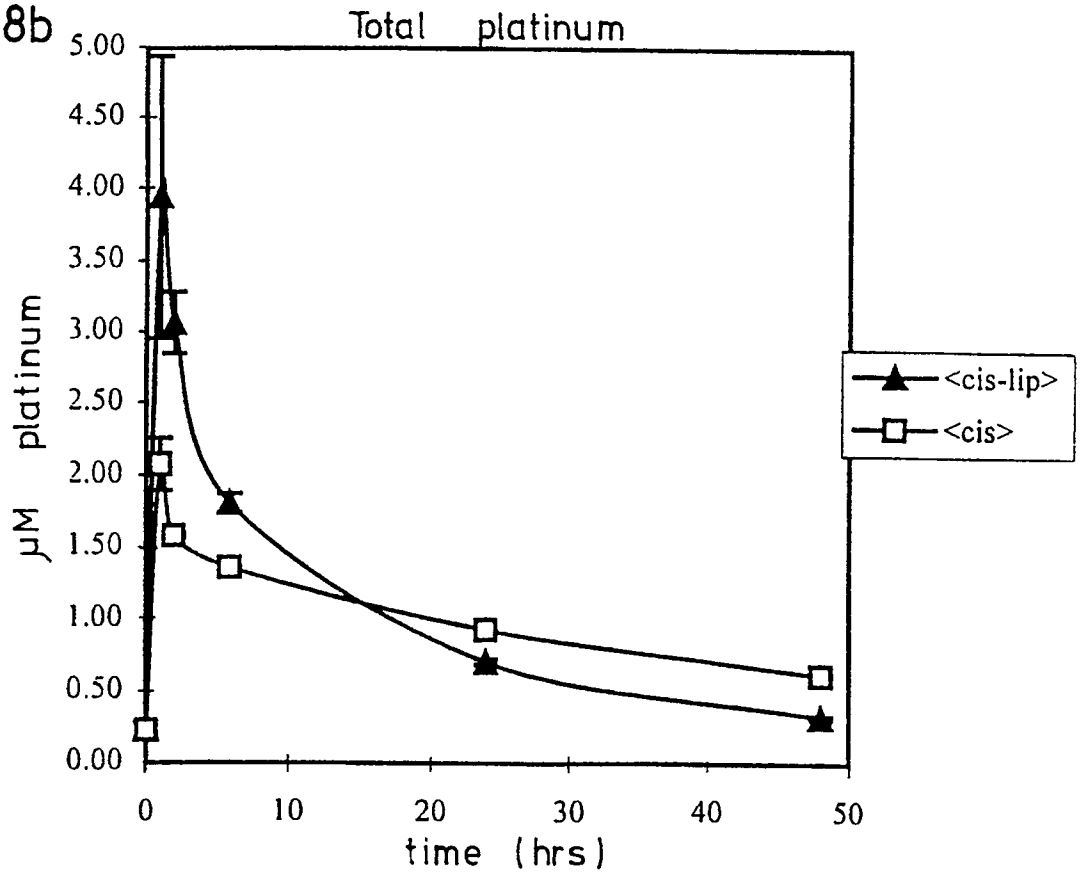

Free platinum
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
µM platinum
0
10
20
30
40
50
time (hrs)
<cis-lip>
<cis>

Fig. 8b


Total platinum
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
µM platinum
0
10
20
30
40
50
time (hrs)
<cis-lip>
<cis>

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/NL 97/00661

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/28 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	G. SPEELMANS ET AL: BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1283, no. 1, 14 August 1996, pages 60-66, XP002040390 cited in the application see page 63, figure 2B,D: DOPS; pages 64-65, discussion, in particular page 65, column 2, lines 24-26 ---	1-5, 7-15,17, 19-24
Y	US 5 059 591 A (A.S. JANOFF ET AL) 22 October 1991 see column 6, A 2.4; column 13, A 4.4; claims 1, 24, 27 --- -/--	1-5, 7-15,17, 19-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1998	12.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Amsterdam, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/NL 97/00661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 15774 A (APHIOS CORP) 30 May 1996 cited in the application see page 5, line 14 - page 6, line 3; page 9, lines 9-11; claims 1, 8, 10, 13, 21	1-5, 7-15, 17-24
A	WO 90 02131 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 8 March 1990 cited in the application see claims 1,14,17,19-24; examples 32,33	1,14,17, 19-24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 97/00661

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 21-23
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern .al Application No
PCT/NL 97/00661

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5059591 A	22-10-91	US 4897384 A	30-01-90
		MX 9203662 A	01-07-92
		CA 1237670 A	07-06-88
WO 9615774 A	30-05-96	AU 4246296 A	17-06-96
		EP 0792143 A	03-09-97
WO 9002131 A	08-03-90	US 5117022 A	26-05-92
		AU 4194889 A	23-03-90